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단안의 각막 신경 절단이 양안의 안표면과 눈물샘에 미치는 효과에 관한 고찰

Bilateral effect of the unilateral corneal nerve cut on both ocular surface and lacrimal gland

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논 문 초 록

외국어초록(Abstract)

Abstract

Bilateral effect of the unilateral corneal nerve cut on both ocular surface and lacrimal gland

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Purpose: To investigate the effect of unilateral cut of corneal nerve on the bilateral ocular surface and tear secretory function.

Methods: 7 week-old female BALB/c mice were divided into control and nerve cutting (NC) groups (n=60). The left cornea was partially incised with a 2.0mm circular trephine, through the upper half of the stromal layer. Lissamine green corneal staining and tear volume measurements were conducted, and corneal whole-mount staining using class III β-tubulin antibody was performed to assess corneal nerves. Flow cytometric analysis for dendritic cells (DCs), $CD4^+$/$CD8^+$ and regulatory T cells and enzyme-linked immunosorbent assay (ELISA) for neuropeptides were performed.

Results: The grading of corneal staining increased in the NC group, while the tear volume decreased over the four weeks. The nerve density decreased in bilateral corneas over two weeks. At day 14, $CD11b^{+}$ or $CD11c^{+}$ DCs and the mature DCs expressing CD86 or MHCII increased in bilateral cornea/conjunctiva. At day28, $CD11c^{+}CD86^{hi}$, $CD11c^{+}MHCII^{hi}$, Th17 and IFN-γ secreting CD8+ T cells highly increased in bilateral draining lymph nodes. $CD4^{+}CD25^{hi}Foxp3^{hi}$ and $CD8^{+}CD25^{hi}Foxp3^{hi}$ regulatory T cells notably increased in spleen. In ELISA, NPY, CGRP and VIP were generally suppressed in the extra-orbital lacrimal glands at day14.

Conclusions: The unilateral corneal nerve severing resulted in activation of the immune cells on the ocular surface and dysregulated lacrimal secretion bilaterally through the bi-directional neuronal signals. It suggests that the unilateral corneal nerve damage may alter immune homeostasis and mechanistically participate in the development of bilateral inflammatory disorder such as dry eye.

Keywords: Corneal nerve; Dry eye; Ocular surface; Lacrimal gland; Ocular immune privilege

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목 차

그 림 목 차

Figures

Supplementary Figure

INTRODUCTION

Dry eye disease (DED) is a multifactorial disease characterized by unstable tear film and ocular surface damage which results in a visual disturbance with ocular discomfort.[1, 2] While the pathogenesis of DED has not been fully established, chronic inflammation was reported to play a prominent role.[3-5] When the cornea is exposed to desiccating stress, tear film becomes a hyperosmolar state and the inflammatory cytokines are released from the ocular surface tissues. Th17 cell is known as a key player in the pathogenesis of DED.[4] Not only the desiccating stress but also the various conditions such as aging, contact lens, laser in situ keratomileusis (LASIK) surgeries and eyelid disorders are able to initiate the immune cascade leading DED.[6-9] Although the injury of the corneal nerve during the LASIK surgery is considered as the main factor, the mechanism of the DED development following the surgery is still unclear.[9]

The cornea has the densest innervation among the peripheral tissues in the human body. The nerve endings mostly derived from trigeminal ganglion form the subbasal nerve plexus just below the corneal epithelium.[10, 11] The function of the corneal nerve is essential to maintain a healthy ocular surface and to regulate the homeostasis of the immune responses.[3, 12] Inflammation is suppressed under neural control, allowing the corneal surface as an immune-privileged site.[13] A recent study reported that circular corneal incision in one eye could abolish the immune privilege of the bilateral ocular surface, leading to a high rate of corneal allograft rejection.[14] Additionally, it was reported that some corneal disease in the unilateral eye could

alter the immune cells, nerve density and cytokine levels in bilateral eyes.[15-17] Based on the previous studies, we evaluated whether the corneal nerve cut in a single eye would have a bilateral effect on 1) immune reaction in ocular surface, 2) lacrimal secretory function and 3) morphological changes of corneal nerve fibers in a murine model.

METHODS

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Seoul National University Biomedical Research Institute (IACUC No. 16-0107-S1A0 and 17-0091-S1A0). Animal experiments were performed in accordance with the ARVO Statement for Use of Animals in Ophthalmic Vision and Research.

Animals

Sixty of 7-week BALB/c female mice were purchased from Orient Bio Inc. (Seongnam, Gyeonggi-do, Korea). The mice were randomly divided into two groups (control and nerve cutting (NC) groups, n=30 per group). Experimental scheme is summarized in **Figure 1A**.

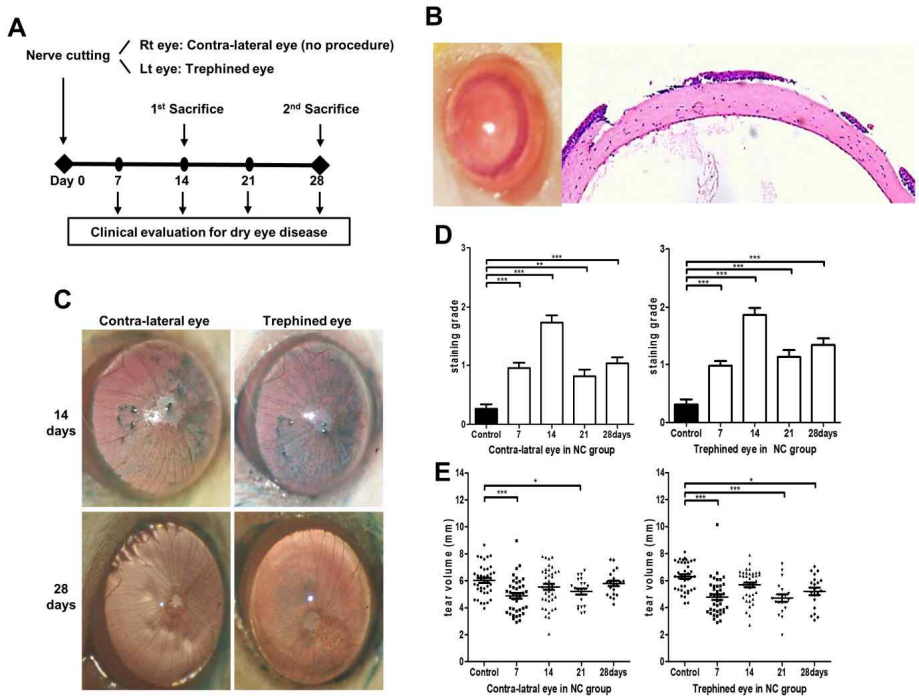


Figure 1. Experimental scheme and examination of corneal epithelial damage and tear volume.

A. Scheme of experiments. 7-week BALB/c female mice were randomly divided into two groups (control and nerve cutting (NC) groups). For the NC group, the left cornea was trephined to sever the corneal nerves. Corneal epithelial staining and tear volume measurements were performed every week for all the four weeks of study period. At day 14 and 28, half of each group was sacrificed for flow cytometry analysis. **B.** To sever the corneal subbasal and stromal nerves, the cornea was partially incised with a 2mm circular punch. After the procedure, histologic examination with hematoxylin and eosin staining

demonstrated that the corneal epithelium and the half of the corneal stroma were incised. **C.** Representative photographs of corneal epithelial staining with lissamine green dye at day 14 and 28. **D.** In the NC group, corneal staining grading was significantly increased in bilateral eyes compared with that of controls for all the four weeks. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by ANOVA) **E.** Tear volume measurements of bilateral eyes were significantly decreased in the NC group for all the study period. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by ANOVA)

Corneal nerve cutting

To sever the subbasal nerve plexus and the stromal nerves of the cornea, the corneal surface of the left eye in the NC group was incised using a circular punch (KAI medical; Kai Europe GmbH, Solingen, Germany) with a 2.0 mm diameter.[14] The punch was applied to the corneal surface and then twisted five to seven times with slight pressure until the epithelium and the half-depth of the stroma were incised (**Figure 1B**), which was adopted from the previous study.[14] At the end of the procedure, a drop of 0.5% levofloxacin eye drop was instilled into the trephined eye. As the nerve cutting was performed only for the left eye, the right and left eyes of the NC group were defined as contra-lateral and trephined eyes, respectively, for the statistical analysis.

Corneal epithelial damage staining

The amount of corneal epithelial damage was evaluated using lissamine green dye (3% Lissamine green B; Sigma-Aldrich, Saint Louis, Missouri, USA). Under anesthesia, one drop of the dye was applied to the ocular surface. The extent of the staining was assessed by a single observer (L.H.K) in a blinded manner using a grading scale as follows; score 0 for no punctuate staining, score 1 when less than one-third of the corneal surface was stained, score 2 when from one-third to two-thirds were stained, score 3 when more than two-thirds were stained.[18] The staining evaluation was performed before the trephination and every week for a total of four weeks of the observation period.

Tear volume measurement

Aqueous tear production was measured by the phenol red thread test. Under anesthesia, a phenol red-impregnated cotton thread (FCI Ophthalmics, Pembroke, MA, USA) was placed at the lower lateral canthus. After 60 seconds, the thread was gently removed and the length of the wet portion (denoted by the red color change) was measured in millimeters.[18] The tear volume evaluation was performed before the nerve cutting and every week for four weeks.

Corneal Whole-Mount Staining

The mice were euthanized by cervical dislocation under deep anesthesia with zoletil, according to the American Veterinary Medical Association Guidelines for the Euthanasia of Animals (2013 Edition). After enucleation, the corneas were freed from the anterior segments. The corneas were fixed for 10 minutes in ice-cold acetone mixed with methanol (1:1) and then rinsed in PBS. The preparations were stored in 2% BSA at 4°C for 24 hours. The tissues were blocked with Dako Protein Block Serum-Free (Dako, Carpinteria, CA, USA) for 10 minutes at room temperature. To identify the corneal nerve fibers, we used neuronal class III β-tubulin antibody (#AB15708A4, Alexa Fluor 488-conjugated, 1:400; Millipore, Billerica, MA, USA). At 4 hours after the applying antibody mixture, the cornea tissues were rinsed with PBS. Four radial incisions were made toward the center of the cornea, and then the flattened tissues were observed using Vectashield containing DAPI (Vector Laboratories, Burlingam, CA, USA).

Flow cytometry

At day 14 and 28 after the corneal nerve cutting, the cornea/conjunctiva, draining lymph nodes, and spleens were extracted and collected. The proportion of mature DCs was determined by measuring the expression of the co-stimulatory molecule (CD86) or major histocompatibility complex (MHC) class II on CD11b or CD11c expressing cells, using flow cytometry. To determine the subset of the effector T cells, the proportion of IFN-γ, IL-17A, CD103, and the CD69 expression on $CD4^+$ or $CD8^+$ T cells were assessed. For regulatory T cells, simultaneous expression of CD25 and Forkhead box protein 3 (Foxp3) on $CD4^+$ or $CD8^+$ T cells were measured.

To get the cell suspensions, the collected lymph nodes and spleens were minced between the frosted ends of two glass slides in RPMI media (WelGENE, Daegu, Korea). The media contained 10% fetal bovine serum and 1% penicillin-streptomycin. The extracted cornea and conjunctiva tissues were cut into small pieces by microscissors and lysed in the RPMI media. Cell suspensions were collected and immunostained with following fluorescence-conjugated anti-mouse antibodies: CD11b (DCs; #11-0112-82, eBioscience, San Diego, CA, USA), CD11c (DCs; #11-0114-82, eBioscience), CD86 and MHC class II (mature DC; #11-0862-82 and #11-5321-82, eBioscience), CD3 (#11-0032-82, eBioscience), CD4 (#11-0042-82, eBioscience), CD8 (#25-0081-82, eBioscience), CD69 (#17-0691-82, eBioscience), CD 103 (#11-1031-82, eBioscience), IFN-γ (#11-7311-41, eBioscience), IL17A (#559502, BD PharmingenTM, San Diego, CA, USA), CD25 and FoxP3 (#17-0251-82 and #12-4771-82, eBioscience, San Diego, CA, USA). The cells were stimulated for 4 hours with 50 ng/mL phorbol myristate acetate and 1 g/mL ionomycin in the

presence of GolgiPlug (BD PharmingenTM, San Diego, CA, USA) for intracellular staining. Using a FACSCanto flow cytometer (BD BioSciences, Mountain View, CA, USA), the fluorescence assays of the cells were performed. With the FlowJo program (Tree Star, Inc., Ashland, OR, USA) data were analyzed.

Enzyme-Linked Immunosorbent Assay

Enzyme-Linked Immunosorbent Assay (ELISA) was performed to quantify the level of neuropeptides, using specific commercially available kits. For peptides extraction, extra-orbital lacrimal glands were minced into small pieces and sonicated in PRO-PREP Protein Extraction Solution (Intron Biotechnology, Seongnam, Korea) on ice. The supernatant was collected after centrifugation at 13,000g for 20 minutes, and assayed for the concentration of substance P (SP; R&D Systems; detection limit: 43.8 pg/ml), calcitonin gene-related peptide (CGRP; phoenix pharmaceuticals; detection limit: 0.16 ng/ml), vasoactive intestinal peptide (VIP; phoenix pharmaceuticals; detection limit: 0.12 ng/ml) and neuropeptide Y (NPY; phoenix pharmaceuticals; detection limit: 0.09 ng/ml) by ELISA according to the manufacturer's protocol.

Statistical Analysis

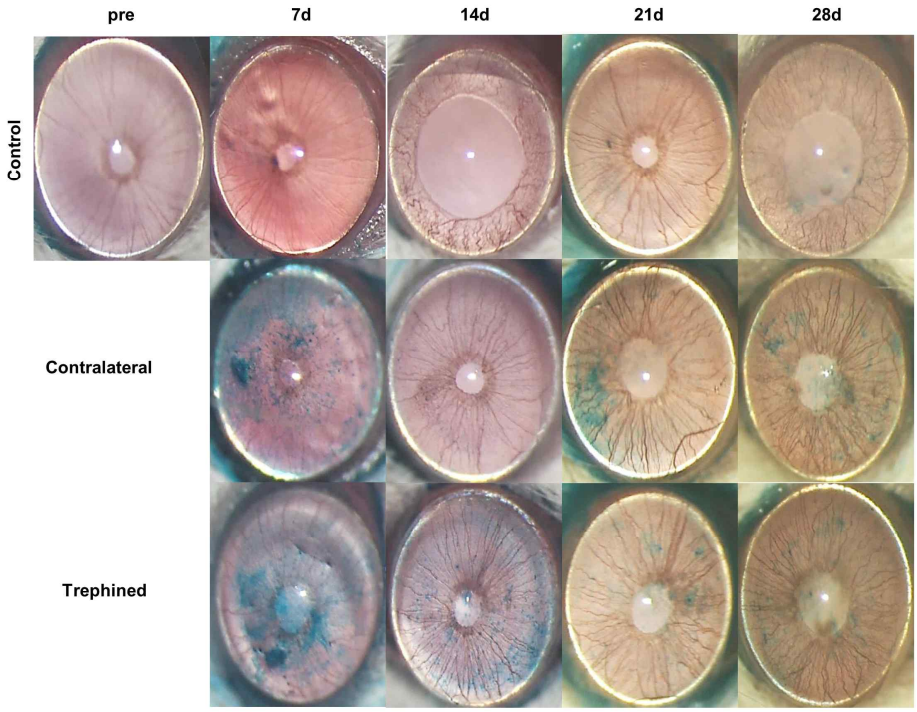
GraphPad Software (GraphPad Prism® Ver 5.01, Inc., La Jolla, CA, USA) was used for statistical tests. Homogeneity of variance was assessed by Levene test. To compare three groups (control, contra-lateral, trephined groups), data were analyzed by the Kruskal-Wallis test. When the null hypothesis was rejected ($p < 0.05$), pairwise comparisons of the groups were performed with

the Mann-Whitney test. Comparison of data from two groups (control and NC groups) was performed with the Mann-Whitney test. Data were presented as the mean ± standard error. Differences were considered significant when $p < 0.05$.

RESULTS

Severing corneal nerves in a single eye induced bilateral DED in a murine model.

We first evaluated whether the severing the corneal nerve in one eye would induce clinical manifestations of DED in bilateral eyes. In the NC group, corneal staining grades were significantly increased in bilateral eyes compared with those in the control group for all the four weeks of the observation (**Figure 1C-D, Supplementary Figure S1**). The difference of the staining grades between the control and NC groups was the peak at day 14. Consistent with the corneal epithelial damage, tear volume was significantly decreased in bilateral eyes in NC group during the study period (**Figure 1E**). The maximal statistical significance of the tear volume measurements was shown on day 7. Taken together, we concluded that unilateral corneal nerve damage would lead to bilateral DED which would persist for at least 28 days.



S1. The representative photographs of corneal epithelial staining with lissamine green dye for control, contra-lateral, and trephine eyes.

Severing corneal nerves in a single eye induced loss of corneal subbasal and stromal nerve in bilateral eyes.

We assessed the state of corneal innervation by double-labeling of nerves and cell nuclei. In the normal cornea, two layers of innervation - stromal nerve network and dense subbasal nerve plexus - were observed (**Figure 2A**). The subbasal nerve bundles run centripetally and formed whorl-like structure at the central area. Immediately after unilateral trephination, focal areas of subbasal nerve dropout began to be observed in the contra-lateral eyes (**Figure 2B**). At day 7, in the trephined eyes, subbasal and stromal nerve fiber loss with dropouts of overlying epithelium was observed in the broad area. (**Figure 2C**). In the contra-lateral eyes, the architecture of the nerve bundles was partially disrupted especially in the corneal center and the whorl-like structure was not observed. At day 14, the area of nerve loss in the trephined cornea became much enlarged (**Figure 2D**). In the contra-lateral eyes, central denervation was more prominent than those at day 7.

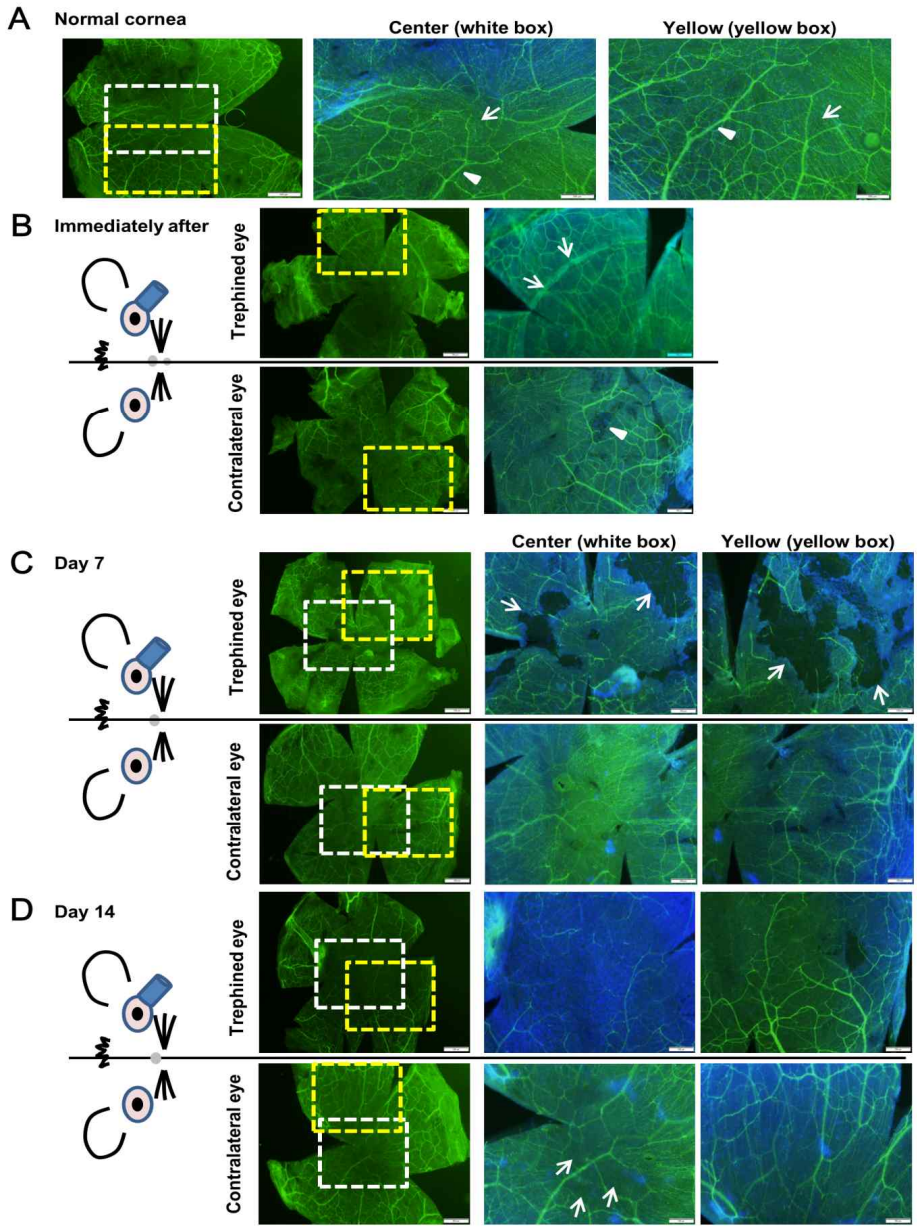


Figure 2. Time-dependent change in the distribution of subbasal and stromal nerve bundles following unilateral corneal trephination.

Representative corneal whole-mount images of immunostaining for class III β-tubulin and epithelial nuclei before (**A**), immediately after (**B**), one (**C**) and two weeks (**D**) from the trephination. Full (x40 magnification; scale bar = 500 μm) and detailed (x100 magnification; scale bar = 200μm) images were taken in each cornea tissue. **A.** In the normal healthy cornea, dense and regular subbasal nerve bundles with central whorl-like appearance (arrows) and deeper stromal nerves (arrowheads) were observed. **B.** Trephined line (arrows) was marked in the trephined eye. Note the presence of patchy-like focal dropout of subbasal nerve bundles (arrowheads) in the contra-lateral eye. **C.** At one week after the unilateral trephination, subbasal and stromal nerve density was remarkedly decreased in the trephined eye. Broad denervation with the patchy-like loss (arrows) was observed in the central and peripheral cornea. In the contra-lateral eye, subbasal and stromal nerve density was also decreased compared with those of normal cornea. Notably, the whorl-like appearance of nerve bundles was not observed in the central area. **D.** At two weeks after the unilateral trephination, the loss of nerve fiber bundles and morphological changes were more extensive. In the trephined eye, subbasal and stromal nerve fiber was not observed in the central and mid-peripheral area. In the contra-lateral eye, central denervation (arrows) was more marked compared with those at one week.

Severing corneal nerves in a single eye enhanced accumulation and maturation of dendritic cells in bilateral cornea/conjunctiva and draining lymph nodes.

Next, we explored the DC population in cornea/conjunctiva and draining lymph nodes using flow cytometry. First, we gated $CD11b^+$ or $CD11c^+$ cell population. Then, the expression of CD86 or MHC class II was assessed for mature DCs (**Figure 3A**). At day 14, the numbers of $CD11b^+$ and $CD11c^+$ cells in bilateral cornea/conjunctiva increased in NC group compared with those of control group, with significant and marginal differences, respectively (p = 0.0249, 0.0548, respectively) (**Figure 3B**). Also, mature DCs expressing CD86 or MHC class II were significantly accumulated more in bilateral eyes of NC group compared with controls. However, at day 28, DC population in cornea/conjunctiva didn't show any significant difference among the groups, except the $CD11c^+$ cells (**Figure 3C**). Meanwhile, in draining lymph nodes at day 28, mature DCs such as $CD11c^+CD86^{hi}$ and $CD11c^+MHCII^{hi}$ cells notably increased bilaterally with moderate and significant differences, respectively (p = 0.0562, 0.0010, respectively) (**Figure 4**).

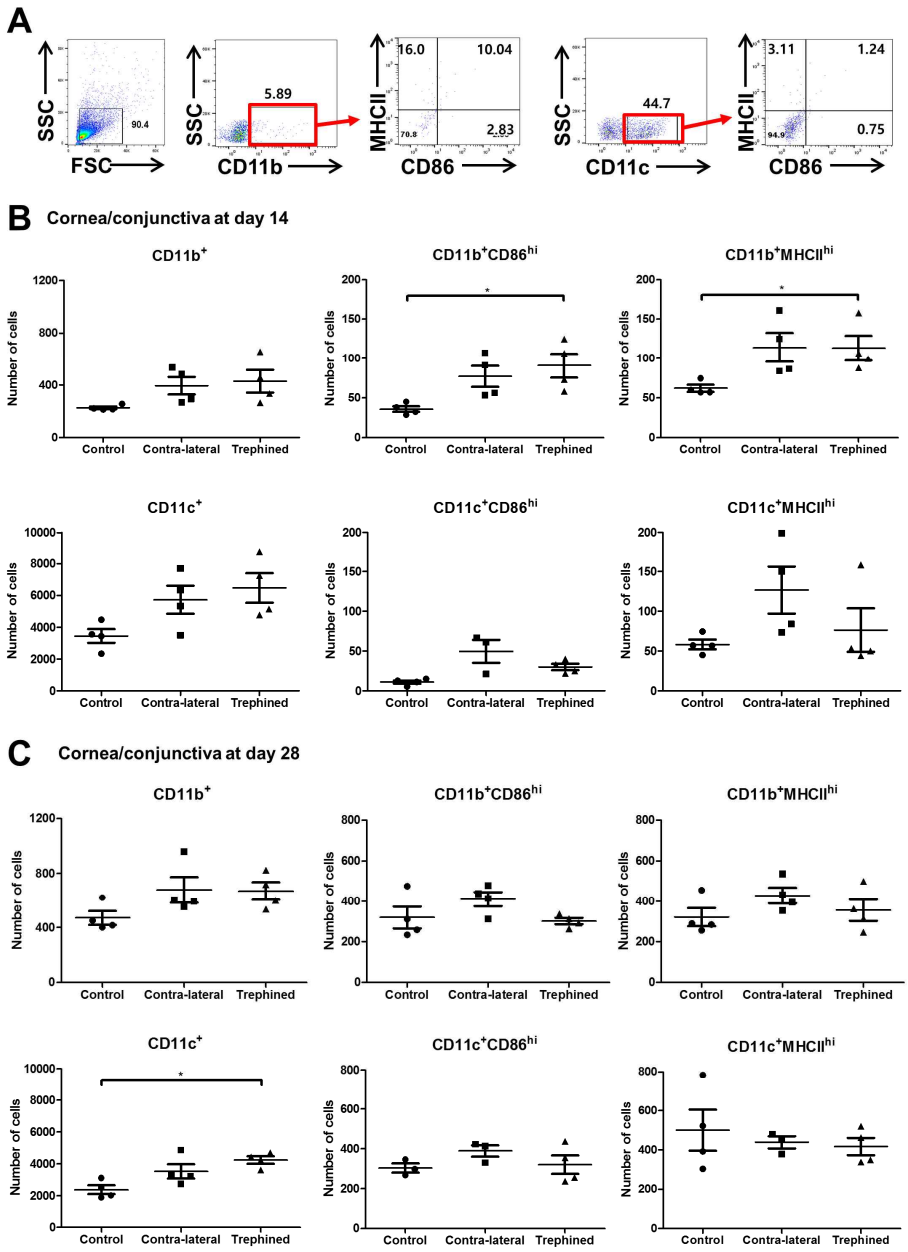


Figure 3. The result of flow cytometry analysis for dendritic cells in cornea/conjunctiva.

A. For dendritic cells, $CD11b^+$ or $CD11c^+$ cells were counted. For mature form, CD86 or MHC class II expression on $CD11b^+$ or $CD11c^+$ cells were gated. **B.** At day 14, the number of CD11b or CD11c positive cells and the mature forms were increased in bilateral cornea/conjunctiva in nerve cutting group. Especially $CD11b^+CD86^{hi}$ or $CD11b^+MHCII^{hi}$ cells were significantly increased. ($*p < 0.05$, by Kruskal Wallis test) **C.** At day 28, except $CD11c^+$ cells, the number of dendritic cells didn't show statistical difference among the groups. ($*p < 0.05$, by Kruskal Wallis test)

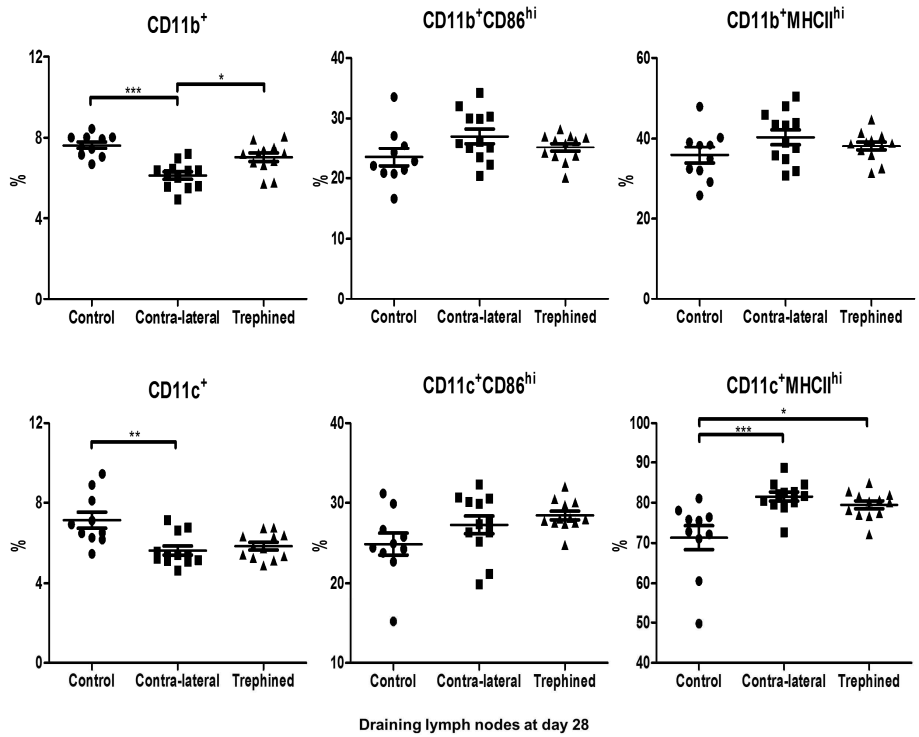


Figure 4. The result of flow cytometry analysis for dendritic cells in draining lymph nodes.

At day 28, the expression of CD86 or MHC class II on $CD11b^+$ cells was not different among the groups. However, the CD86 expression on $CD11c^+$ cells increased in contra-lateral and trephined eyes compared with that of control eyes with a moderate significance ($p = 0.0562$). MHC class II was also highly expressed in both contra-lateral and trephined eyes than that of controls with a statistical significance ($p = 0.0010$). ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, by Kruskal Wallis test)

Unilateral corneal nerve cutting activated IFN-γ producing $CD8^+$ T cell in draining lymph nodes.

Then, we evaluated the subset of resident memory or effector T cell population in bilateral cornea/conjunctiva and draining lymph nodes. First, we gated CD103 and CD69 expressing T cells in cornea/conjunctiva tissue (**Figure 5A**). At day 14, the proportion of $CD4^+CD69^{hi}$ cell increased significantly in bilateral eyes of the NC group (p = 0.0249) (**Figure 5B**). However, at day 28, CD69 expression didn't show significant differences among the groups (**Figure 5C**). In draining lymph nodes, we measured IL-17A or IFN-γ expression on $CD4^+$ or $CD8^+$ T cells (**Figure 6A**). At day 28, the proportion of Th17 cell significantly increased in the NC group (p = 0.0247) (**Figure 6B**). Notably, IL-17A expression on $CD8^+$ T cells decreased in the NC group, while IFN-γIexpression increased with statistical significances (p = 0.0102, 0.0169, respectively).

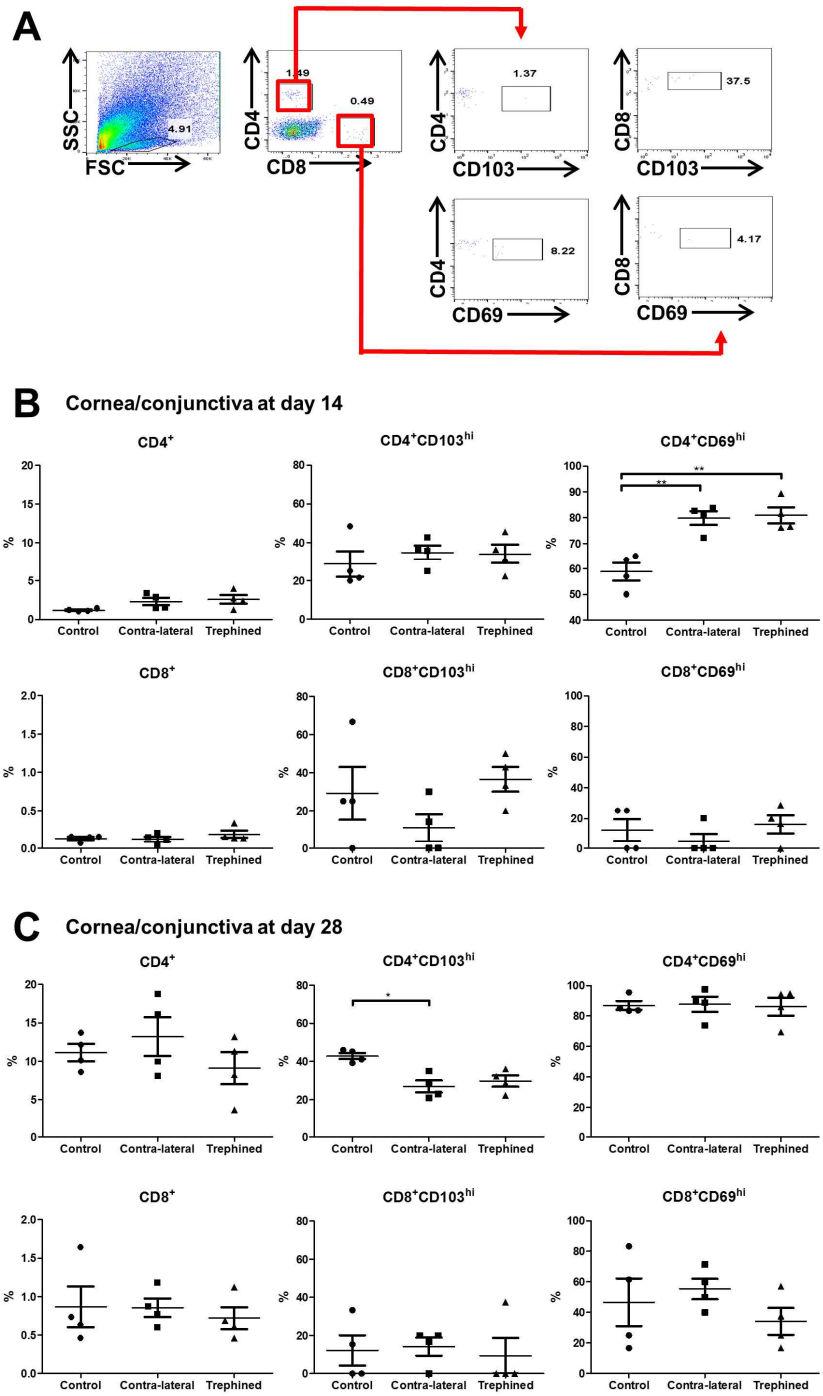

A
SSC
FSC
4.91
CD4
CD8
1.49
0.49
CD4
CD103
1.37
CD8
CD103
37.5
CD4
CD69
8.22
CD8
CD69
4.17
B
Cornea/conjunctiva at day 14
CD4+
CD4+CD103hi
CD4+CD69hi
CD8+
CD8+CD103hi
CD8+CD69hi
%
Control
Contra-lateral
Trephined
**
**
C
Cornea/conjunctiva at day 28
CD4+
CD4+CD103hi
CD4+CD69hi
CD8+
CD8+CD103hi
CD8+CD69hi
%
Control
Contra-lateral
Trephined
*

Figure 5. The result of flow cytometry analysis for effector T cells in cornea/conjunctiva.

A. For gating resident and activated effector T cells in cornea/conjunctiva, the expression of CD103 and CD69 on $CD4^+$ or $CD8^+$ T cells was assessed, respectively. **B.** At day 14, $CD4^+$ T cell and its activated form expressing CD69 increased in cornea/conjunctiva in nerve cutting group. (p = 0.0388, 0.0249, respectively.) **C.** At day 28, the proportion of effector T cells in cornea/conjunctiva didn't increase in nerve cutting group. ($*p < 0.05$, by Kruskal Wallis test)

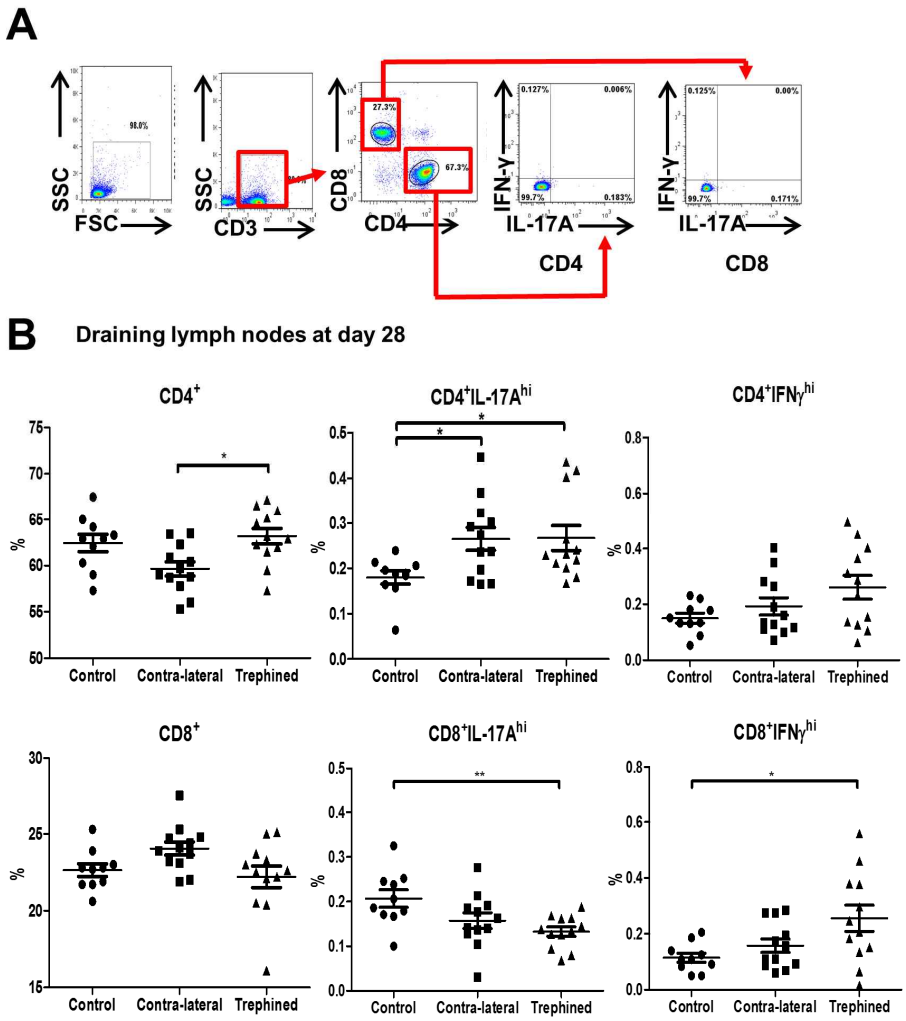


Figure 6. The result of flow cytometry for effector T cells in draining lymph nodes.

A. For the effector $CD4^+$ or $CD8^+$ T cells in draining lymph nodes, the expression of IL-17A and IFN-γ was assessed. B. At day 28, Th17 cells significantly increased in bilateral draining lymph nodes. (p = 0.0247) Interestingly, IFN-γ^+ cells also

increased in draining lymph nodes of trephined eyes in nerve cutting group. (*$p < 0.05$, **$p < 0.01$, by Kruskal Wallis test)

Severing corneal nerves in a single eye enhanced the generation of regulatory T cells in the spleen.

Along with the assessment of the immune cell subsets in cornea/conjunctiva and draining lymph nodes, we analyzed splenic cell population at day 14 and 28. To assess the regulatory T cell population, we gated the concomitant expression of CD25 and Foxp3 on $CD4^+$ or $CD8^+$ T cells (**Figure 7A**). At day 14, the proportion of regulatory T cell was not significantly different between control and NC groups (**Figure 7B**). However, at day 28, the proportion of splenic $CD4^+CD25^{hi}Foxp3^{hi}$ and $CD8^+CD25^{hi}Foxp3^{hi}$ cell increased in NC group compared with controls (p = 0.0231, = 0.1231, respectively) (**Figure 7C**).

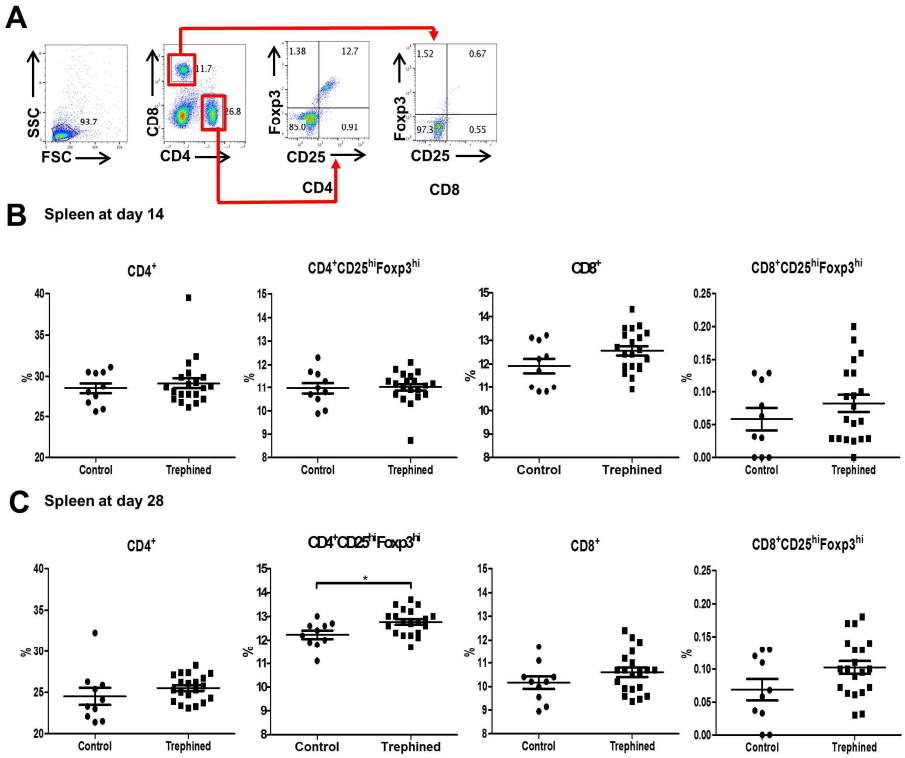


Figure 7. The result of flow cytometry for regulatory T cells in the spleen.

A. For regulatory T cells, concomitant expression of CD25 and Forkhead box protein 3 (Foxp3) on CD4 or CD8 positive T cells was assessed. **B.** At day 14, regulatory T cells in the spleen didn't show a significant difference between the control and the nerve cutting groups. **C.** At day 28, splenic regulatory T cells increased in nerve cutting group compared with controls. Especially, $CD4^{+}CD25^{hi}Foxp3^{hi}$ cells showed significant differences. ($p = 0.0231$) ($*p < 0.05$, by Kruskal Wallis test)

Severing corneal nerves in a single eye would alter the neuropeptide level of extra-orbital lacrimal glands.

The level of SP, NPY, CGRP and VIP of extra-orbital lacrimal glands was assessed by ELISA. Immediately after the corneal trephination, the VIP level was significantly increased only in trephined eyes (p = 0.0276) (**Figure 8A**). At day 7, CGRP level was significantly increased in contra-lateral and trephined eyes compared with that of the control group (p = 0.0078) (**Figure 8B**). At day 14, CGRP level significantly decreased in the contra-lateral eyes (p = 0.0144) and VIP level was suppressed in contra-lateral and trephined eyes (p = 0.0089) (**Figure 8C**).

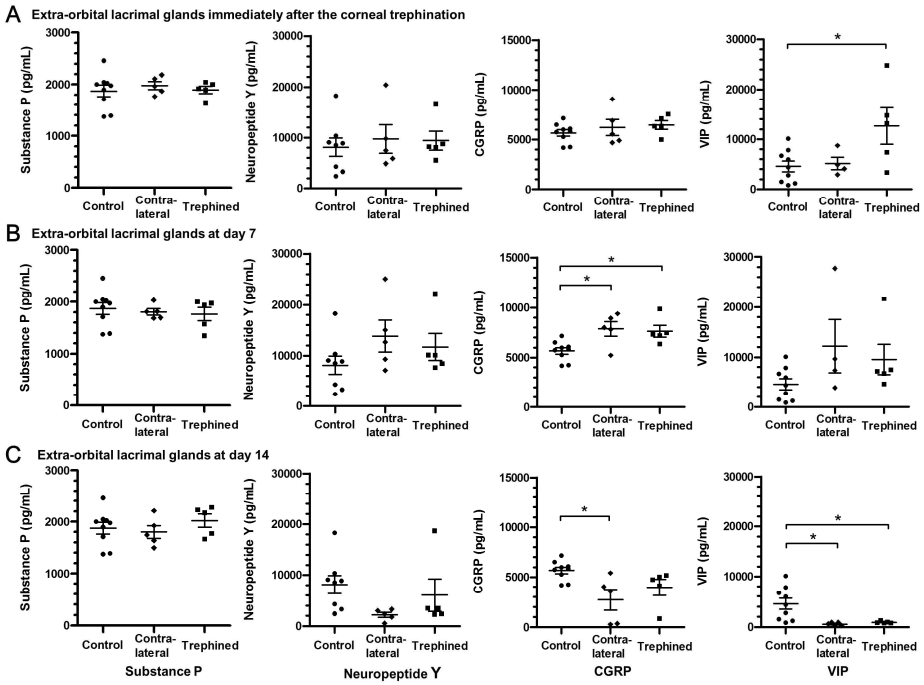


Figure 8. The result of ELISA for neuropeptide level in the extra-orbital lacrimal glands.

The level of neuropeptides was compared among the control, contra-lateral and trephined eyes. **A.** Immediately after the unilateral corneal trephination, VIP level significantly increased in the trephined eye (p = 0.0276), while the level of SP, NPY and CGRP was not different among the groups. **B.** At day 7, CGRP level significantly increased in the contra-lateral and trephined eyes compared with that of the control group (p = 0.0078), while the other neuropeptides were not different among the groups. **C.** At day 14, CGRP level significantly decreased in the contra-lateral eyes (p = 0.0144) and VIP level significantly

decreased in the contra-lateral and trephined eyes (p = 0.0089), while the level of SP and NPY didn't show significant changes. (*p < 0.05, by ANOVA)

DISCUSSION

Our study shows that unilateral circumferential corneal nerve severing induces 1) bilateral ocular surface inflammation mediated by anterograde nerve degeneration and 2) bilateral hypofunction of lacrimal glands by retrograde neural pathway, as presumptively illustrated in **Figure 9**.

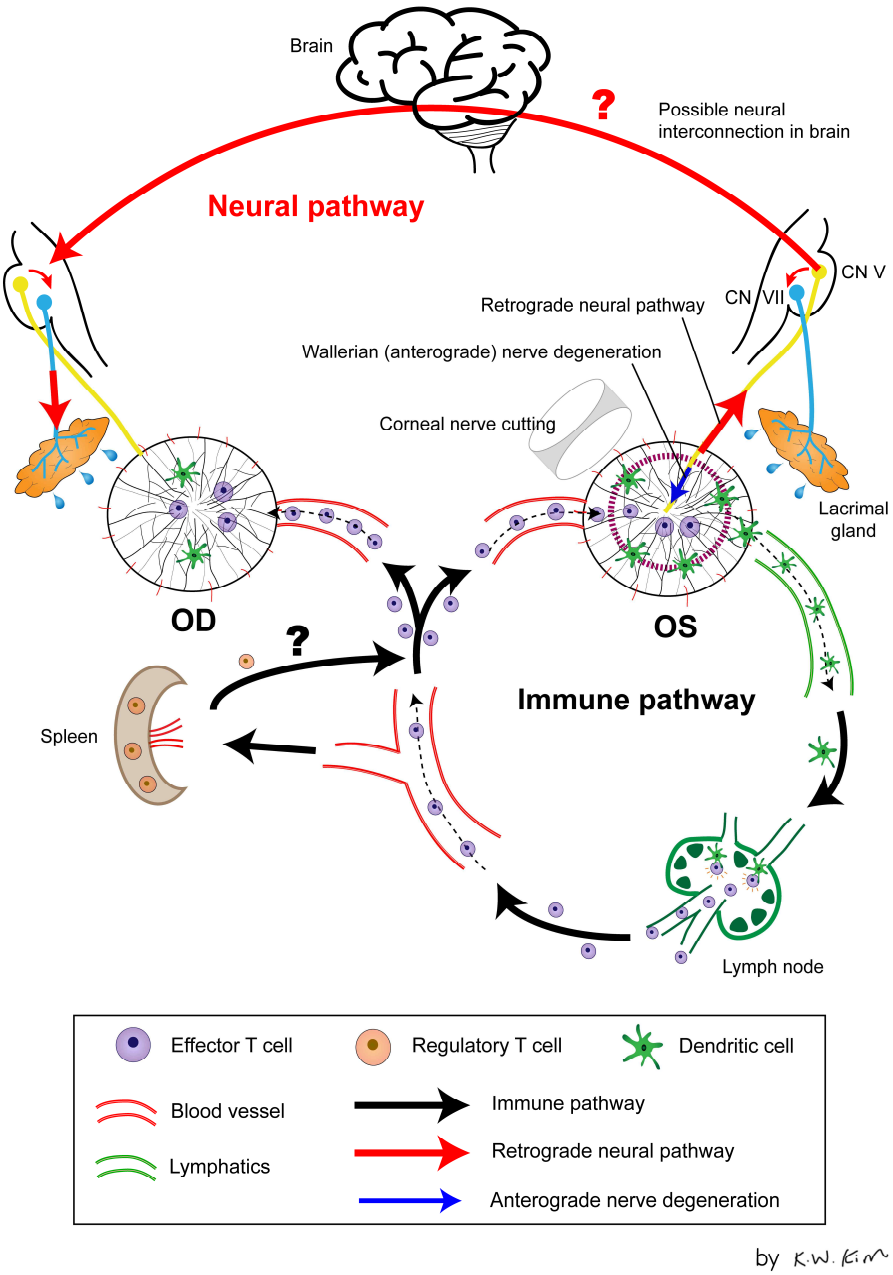


Figure 9. The hypothetical illustration showing scenario of how the unilateral corneal nerve cut may affect bilateral

ocular surface through the immunologic and neural pathway.

This schematic illustration summarizes the effect of the unilateral corneal cutting on the bilateral ocular surface and lacrimal glands. When the corneal subbasal and stromal nerves are cut by the trephination in one eye, the signals formed from the injurious stimuli transmitted to the bidirectional way. The anterograde nerve degeneration may increase the immune response of corneal tissue, thereby generating mature dendritic cells. The mature dendritic cells migrating to the draining lymph nodes can activate effector lymphocytes, and the effector cells arrive ipsilateral and contra-lateral ocular surface through the blood vessels. Meanwhile, the retrograde centripetal nerve signals to the brainstem nucleus may cross-over and alter the bilateral lacrimal gland secretory function.

Since the subbasal nerve bundles run from the periphery to the center in a perpendicular direction, we assumed that the subbasal nerve fibers were completely severed by the mid-peripheral circular trephination.[19] When the trephination was restricted to the corneal epithelium, the subbasal nerve fibers would rapidly recover to its original density within a few days.[20] However, the recovery of the nerve fibers was delayed in this study, as the subbasal and stromal nerves had been cut together by the trephination through the upper half of the stromal layer.[21] As expected, the subbasal and stromal nerve density gradually decreased during the fourteen days of the observation period, rather than recovered. Surprisingly, the corneal nerve density in the opposite eye also decreased, which was not directly injured.

Antidromically-evoked depolarization enables the release of neuropeptides contained in the corneal nerve endings that were not directly exposed to the stimulus, leading extended inflammation to neighbor areas, which is called “neurogenic inflammation”.[22] This study did not prove local neuroinflammation. On the contrary, this study showed some of the immune-activated cells traveled to the draining lymph nodes and reached out to the contra-lateral ocular surface. The bilateral nerve density reduction may be explained by the activation of the immune response mediated by Wallerian nerve degeneration in the trephined eye. When a nerve fiber is injured, the distal part of the axons become degenerated as called Wallerian degeneration.[23, 24] During this process, degenerated axons are removed by Schwann cells, macrophages, monocytes, or neutrophils.[23] In the inflammatory environment, the DCs become mature, expressing MHC class II and costimulatory molecules.

The mature DCs egress from the cornea to the draining lymph nodes and activate effector lymphocytes.[25, 26] The mature DCs and effector lymphocytes may migrate to the bilateral ocular surface through the systemic blood circulation. The effector cells that arrive at the bilateral cornea may degenerate corneal nerves further. This hypothesis is supported by the accumulation of mature DCs in bilateral ocular surface and draining lymph nodes, and an increase of effector T cells in bilateral draining lymph nodes, shown in this study. Meanwhile, as the inflammatory tone increased in the immune-privileged site, it was expected that the efforts to return immune homeostasis would be accompanied as well.[27] Accordingly, the regulatory T cells increased in the spleen at day 28 can be considered as a possible compensatory reaction.

In addition to the immune response triggered by the anterograde nerve degeneration, the retrograde signals transmitted to the contra-lateral eye through the centripetal neural interconnection may contribute to the bilateral efferent nerve alteration. Given that the trigeminal nuclei may project the nerve fibers to both sides with the axons crossing between bilateral dorsal horns through the dorsal commissure[16, 28], retrograde nerve signals from the corneal afferent nerve injury may mediate bilateral inflammatory responses leading corneal nerve degeneration as well as the altered secretory function of the lacrimal glands in both eyes. This mechanism may also explain bilateral corneal nerve alteration observed in the unilateral ocular disorder such as herpes zoster ophthalmicus and herpes simplex keratitis, described in the previous studies.[16, 17] Unilateral corneal trephination reduced the bilateral tear volume significantly for more than 28 days. This tear reduction may be explained by the altered

parasympathetic tone innervating lacrimal glands in response to the afferent nerve injury. Having the possible existence of bilateral projecting trigeminal nerves, the afferent nerve impulses propagated to the centripetal direction may stimulate the crossing nerve fibers, resulting in changes of CGRP and VIP releases from bilateral efferent autonomic fibers. Stimulation of either parasympathetic or sympathetic nerves release neurotransmitters that regulate secretion of proteins, electrolytes or water in lacrimal glands.[29, 30] Accordingly, both VIP, as a parasympathetic neurotransmitter, and Neuropeptide Y, as a sympathetic neurotransmitter, contribute to the secretion of lacrimal glands.[29, 30] In this study, statistically significant decrease of VIP in both lacrimal glands seems to be involved in reduction of bilateral tear secretion. Neuropeptide Y tended to decrease at 14 days in the contra-lateral lacrimal gland, which was statistically insignificant. The alteration of the sympathetic pathway is possible, but it is still inconclusive. The second explanation for the bilateral tear volume reduction may be related to the diminished afferent inputs resulted from the extensive bilateral sensory neural loss. The lacrimal functional unit consisting of the ocular surface, lacrimal gland and interconnecting innervation finely controls the tear secretion.[31, 32] If any point of this unit is compromised, tear production is impeded.[33] Taken together, both centripetal stimulation of trigeminal nerves that project bilaterally to trigeminal brainstem nuclei and systemic activation of the immune system seems to act on ocular and neural structures of both sides. Further studies should undergo to reveal the role of each neuropeptide following the corneal nerve injury in the future.

Because of the short experimental period, we do not know when the sensory nerve hypofunction would recover back to normal. In the previous LASIK studies, the corneal sensation was reduced till 3 weeks to 9 months[34], or nerve density didn't recover during the two-years.[9, 35] Therefore, the nerve attenuation may persist for a long time in this study.

In the previous studies, several animal models have been introduced to evaluate the morphological alteration of the corneal nerves following the trigeminal nerve injury by transecting of ciliary nerves [36] or electrolysis of the trigeminal nerve.[37] However, we intended to evaluate the effect of corneal nerve damage presumably accompanied in LASIK, cataract surgery or DED, and thereby corneal trephination was adopted. Although the injured branches were more distal part of trigeminal nerves in our study, bilateral reduction of nerve density corresponds well with the previous study.[36] Notably, in similar with the previous study,[36] the nerve degeneration in the contra-lateral eyes was restricted in the central cornea. The disparity of nerve degeneration between the central and peripheral cornea may be originated from the differences of the nerve fiber structure.[19] Given that the main nerve fibers are unmyelinated nerves in the central cornea and the unmyelinated nerves degenerate faster from the distal part than myelinate nerves, it explains that the neural attenuation was marked in the central cornea.

This study was limited because 1) it is an observational study and 2) structural changes of the gland atrophy or neural alteration in the lacrimal gland were not evaluated. Nevertheless, we believe it is worthy of notice by revealing bilateral

substantial changes in lacrimal glands and immune cell profiles in the conjunctiva/cornea through the circumferential unilateral nerve cut.

In summary, unilateral corneal nerve cut may have an effect on both bilateral ocular surface and bilateral lacrimal glands through the bi-directional nerve stimulation. Anterograde Wallerian degeneration may activate the adaptive immune response and retrograde central stimulation may alter the secretory function of lacrimal function, leading bilateral tear volume reduction and ocular surface inflammation. Therefore, the unilateral corneal nerve injury may mechanistically participate in the development of immune-inflammatory disorder such as DED.

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REFERENCES

1. Tsubota K, Yokoi N, Shimazaki J, et al. New Perspectives on Dry Eye Definition and Diagnosis: A Consensus Report by the Asia Dry Eye Society. *Ocul Surf* 2017;15:65-76.
2. Craig JP, Nichols KK, Akpek EK, et al. TFOS DEWS II Definition and Classification Report. *Ocul Surf* 2017;15:276-283.
3. Stevenson W, Chauhan SK, Dana R. Dry eye disease: an immune-mediated ocular surface disorder. *Arch Ophthalmol* 2012;130:90-100.

4. Chauhan SK, Dana R. Role of Th17 cells in the immunopathogenesis of dry eye disease. *Mucosal Immunol* 2009;2:375-376.
5. Pflugfelder SC, Solomon A, Stern ME. The diagnosis and management of dry eye: a twenty-five-year review. *Cornea* 2000;19:644-649.
6. Gomes JAP, Azar DT, Baudouin C, et al. TFOS DEWS II iatrogenic report. *Ocul Surf* 2017;15:511-538.
7. Stapleton F, Alves M, Bunya VY, et al. TFOS DEWS II Epidemiology Report. *Ocul Surf* 2017;15:334-365.
8. Oh HJ, Park YG, Yoon KC. Changes of ocular surface and tear film in patients with pinguecula and pterygium. *Journal of the Korean Ophthalmological Society* 2006;47:717-724.
9. Ang RT, Dartt DA, Tsubota K. Dry eye after refractive surgery. *Curr Opin Ophthalmol* 2001;12:318-322.
10. Muller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents and function. *Exp Eye Res* 2003;76:521-542.
11. Tuominen IS, Konttinen YT, Vesaluoma MH, Moilanen JA, Helinto M, Tervo TM. Corneal innervation and morphology in primary Sjogren's syndrome. *Invest Ophthalmol Vis Sci* 2003;44:2545-2549.
12. Blanco T, Saban DR. The cornea has “the nerve” to encourage immune rejection. *American Journal of Transplantation* 2015;15:1453-1454.
13. Niederkorn JY. The immune privilege of corneal grafts. *J Leukoc Biol* 2003;74:167-171.
14. Paunicka KJ, Mellon J, Robertson D, Petroll M, Brown JR, Niederkorn JY. Severing corneal nerves in one eye induces

sympathetic loss of immune privilege and promotes rejection of future corneal allografts placed in either eye. *Am J Transplant* 2015;15:1490-1501.

15. Yamaguchi T, Hamrah P, Shimazaki J. Bilateral Alterations in Corneal Nerves, Dendritic Cells, and Tear Cytokine Levels in Ocular Surface Disease. *Cornea* 2016;35 Suppl 1:S65-s70.

16. Hamrah P, Cruzat A, Dastjerdi MH, et al. Unilateral herpes zoster ophthalmicus results in bilateral corneal nerve alteration: an in vivo confocal microscopy study. *Ophthalmology* 2013;120:40-47.

17. Hamrah P, Cruzat A, Dastjerdi MH, et al. Corneal sensation and subbasal nerve alterations in patients with herpes simplex keratitis: an in vivo confocal microscopy study. *Ophthalmology* 2010;117:1930-1936.

18. Kim YJ, Ryu JS, Park SY, et al. Comparison of Topical Application of TSG-6, Cyclosporine, and Prednisolone for Treating Dry Eye. *Cornea* 2016;35:536-542.

19. He J, Bazan HE. Neuroanatomy and Neurochemistry of Mouse Cornea. *Invest Ophthalmol Vis Sci* 2016;57:664-674.

20. Mo J, Neelam S, Mellon J, Brown JR, Niederkorn JY. Effect of Corneal Nerve Ablation on Immune Tolerance Induced by Corneal Allografts, Oral Immunization, or Anterior Chamber Injection of Antigens. *Invest Ophthalmol Vis Sci* 2017;58:137-148.

21. Pajoohesh-Ganji A, Pal-Ghosh S, Tadvalkar G, Kyne BM, Saban DR, Stepp MA. Partial denervation of sub-basal axons persists following debridement wounds to the mouse cornea. *Lab Invest* 2015;95:1305-1318.

22. Belmonte C, Acosta MC, Gallar J. Neural basis of

sensation in intact and injured corneas. *Exp Eye Res* 2004;78:513-525.

23. Rotshenker S. Wallerian degeneration: the innate-immune response to traumatic nerve injury. *J Neuroinflammation* 2011;8:109.

24. Stoll G, Griffin J, Li CY, Trapp B. Wallerian degeneration in the peripheral nervous system: participation of both Schwann cells and macrophages in myelin degradation. *Journal of neurocytology* 1989;18:671-683.

25. Hamrah P, Liu Y, Zhang Q, Dana MR. Alterations in corneal stromal dendritic cell phenotype and distribution in inflammation. *Arch Ophthalmol* 2003;121:1132-1140.

26. Liu Y, Hamrah P, Zhang Q, Taylor AW, Dana MR. Draining lymph nodes of corneal transplant hosts exhibit evidence for donor major histocompatibility complex (MHC) class II-positive dendritic cells derived from MHC class II-negative grafts. *J Exp Med* 2002;195:259-268.

27. Hori J. Mechanisms of immune privilege in the anterior segment of the eye: what we learn from corneal transplantation. *J Ocul Biol Dis Infor* 2008;1:94-100.

28. Pfaller K, Arvidsson J. Central distribution of trigeminal and upper cervical primary afferents in the rat studied by anterograde transport of horseradish peroxidase conjugated to wheat germ agglutinin. *J Comp Neurol* 1988;268:91-108.

29. Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. *Prog Retin Eye Res* 2009;28:155-177.

30. Sabatino F, Di Zazzo A, De Simone L, Bonini S. The Intriguing Role of Neuropeptides at the Ocular Surface. *Ocul Surf*

2017;15:2-14.
31. Tiwari S, Ali MJ, Vemuganti GK. Human lacrimal gland regeneration: Perspectives and review of literature. *Saudi J Ophthalmol* 2014;28:12-18.
32. Lambiase A, Micera A, Sacchetti M, Cortes M, Mantelli F, Bonini S. Alterations of tear neuromediators in dry eye disease. *Arch Ophthalmol* 2011;129:981-986.
33. Stern ME, Gao J, Siemasko KF, Beuerman RW, Pflugfelder SC. The role of the lacrimal functional unit in the pathophysiology of dry eye. *Exp Eye Res* 2004;78:409-416.
34. Chuck RS, Quiros PA, Perez AC, McDonnell PJ. Corneal sensation after laser in situ keratomileusis. *J Cataract Refract Surg* 2000;26:337-339.
35. Moilanen JA, Holopainen JM, Vesaluoma MH, Tervo TM. Corneal recovery after LASIK for high myopia A 2-year prospective confocal microscopic study. *British Journal of Ophthalmology* 2008;92:1397-1402.
36. Yamaguchi T, Turhan A, Harris DL, et al. Bilateral nerve alterations in a unilateral experimental neurotrophic keratopathy model: a lateral conjunctival approach for trigeminal axotomy. *PloS one* 2013;8:e70908.
37. Ferrari G, Chauhan SK, Ueno H, et al. A novel mouse model for neurotrophic keratopathy: trigeminal nerve stereotactic electrolysis through the brain. *Invest Ophthalmol Vis Sci* 2011;52:2532-2539.

국문요약 (국문초록)

요약

단안의 각막 신경 절단이 양안의 안표면과 눈물샘에 미치는 효과에 관한 고찰

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목적: 단안의 각막 신경 절단이 양안의 안표면과 눈물 분비 기능에 미치는 영향을 연구하고자 한다.

방법: 7주령의 female BALB/c 쥐 60마리를 대조군과 신경절단군으로 나누었다. 신경절단군의 좌안 각막을 지름 2.0mm의 원형 절제기(trephine)로 각막 기질층 상부 1/2 깊이로 절제하였다. Lissamine green 각막상피 염색을 시행하였고 눈물량을 측정하였으며, 각막 신경을 평가하기 위해 class III β-tubulin antibody 를 이용해 각막의 온조직표본고정 (whole-mount) 염색을 시행하였다. 유세포분석을 통해 수지상세포, $CD4^+/CD8^+$ T 세포, 조절 T 세포를 분석하였고, 효소면역분석(ELISA)을 이용해 신경펩티드(neuropeptide)를 분석하였다.

결과: 각막상피 염색정도는 대조군에 비해 신경절단군의 양안에서

4주간 유의하게 증가하였고 눈물량은 신경절단군 양안에서 유의하게 감소하였다. 각막 신경 밀도는 양안 모두에서 2주동안 감소하였다. 14일째 희생하여 시행한 각막 및 결막의 유세포분석에서 $CD11b^{+}$, $CD11c^{+}$ 수지상세포와 $CD11c^{+}CD86^{hi}$, $CD11c^{+}MHCII^{hi}$ 세포가 양안 모두에서 증가하였다. 28일째 희생하여 시행한 배액림프절의 유세포분석에서 $CD11c^{+}CD86^{hi}$, $CD11c^{+}MHCII^{hi}$ 세포와 Th17과 IFN-γ를 분비하는 $CD8^{+}$ T 세포가 양안에서 모두 증가해있었다. 비장에서 시행한 유세포분석에서 $CD4^{+}CD25^{hi}Foxp3^{hi}$, $CD8^{+}CD25^{hi}Foxp3^{hi}$ 조절 T 세포가 증가해있었다. 실험 14일째 안와밖(extra-orbital) 눈물샘에서 시행한 효소면역분석에서 NPY, CGRP, VIP는 대체로 억제되어 있었다.

결론: 단안의 각막 신경 절단은, 이로 인해 촉발되는 양방향의 신경 신호 전달을 통해 안표면의 면역 세포들의 활성화와 양측 눈물샘에 대한 조절장애를 발생시킬 것이다. 이는 단안의 각막 신경의 손상이 면역 항상성을 변화시키고, 건성안과 같은 양안의 염증성 질환의 발생의 기전에 영향을 미칠 가능성을 시사한다.

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주요어 : 각막신경; 건성안; 안표면; 눈물샘; 안면역관용

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